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TITLE: The Role of Estrogen Receptor  $\alpha$  K303R Mutation in  
Breast Cancer Metastasis

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13. ABSTRACT (Maximum 200 Words) The activity of estrogen receptor $\alpha$ is activated by both ligand binding and various modifications including phosphorylation and acetylation. Recently we have isolated a somatic ER $\alpha$ mutation which occurs in the major p300 acetylation site of ER $\alpha$ (Lys 303 to Arg, K303R) in human breast hyperplasias. This mutant is significantly correlated with both breast tumorigenesis and development. We report here that a component of the nucleosome remodeling and histone deacetylase complexes (NuRD), called metastasis-associated protein 1-like 1 (MTA1-L1), is an ER $\alpha$ binding protein both <i>in vivo</i> and <i>in vitro</i> . Also, we found that this interaction is ligand independent and requires the N-terminal region of MTA1-L1 and multiple domains of ER. Furthermore, we show that MTA1-L1 downregulates wild-type ER $\alpha$ , but not the K303R ER $\alpha$ mutation activities in transient transactivation assays. Taken together, our results suggest that the altered ER alpha-containing NuRD function due to the K303R mutation could be a possible mechanism for this hypersensitive phenomenon				
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## INTRODUCTION

It is known that estrogens play a key role in regulating the proliferation of normal breast epithelium, as well as breast cancer, through binding with their cognate receptors (ERs), which belong to a superfamily of nuclear steroid receptors and function as transcriptional factors to mediate the mitogenic effects of estrogens. It has been reported (1,2) that less than 10% normal breast epithelium express ER $\alpha$ , but that about 60% of UDH and 60-70% of breast tumors express ER $\alpha$ , so it is felt that the expression and/or activation of ER influences both breast carcinogenesis and progression. There are two current models for ER activation. One is through its cognate ligand binding, the other involves alternative pathways such as phosphorylation, acetylation, or mutation. The K303R ER mutation isolated in this group(3) is correlated with both breast cancerigenesis and development, also this mutation disrupts the major acetylation site of ER, so that we proposed to study the effect of an HDAC-associated protein, MTA-L1 mediated deacetylation on wild-type ER $\alpha$  function, as well as study the effects of expressing the K303R mutation on breast cancer cell metastatic behavior in the following **Specific Aims**:

**Aim 1.** To elucidate the relationship between MTA-L1 expression, and ER $\alpha$  protein acetylation as well as ER $\alpha$  activity: We have already shown an *in vitro* interaction between wild-type ER $\alpha$  and MTA-L1 using GST-pull down experiments. To detect the potential significance of this interaction, we will examine for *in vivo* interactions as well as the effects of ER $\alpha$  agonists and antagonists on this interaction using further GST-pull down and co-immunoprecipitation assays. The effects of MTA-L1 expression on ER $\alpha$  transcriptional activity will also be determined using transient transactivation assays and estrogen-responsive reporter assays. The effects of MTA-L1 expression on ER $\alpha$  acetylation status will be determined using *in vitro* deacetylation assays.

**Aim 2.** To study the metastatic process influenced or driven by the K303R ER $\alpha$  mutation, and to develop a somatic knock-out model of the ER $\alpha$  gene in the T47D cell line to study the *in vivo* effects of the K303R mutation. A somatic knock-out of the human ER $\alpha$  gene will be performed using standard approaches targeted to the amino region or the DNA binding domain. The functional significance of the ER $\alpha$  mutation will first be determined using *in vitro* motility and invasion assays of the knock-out cell line transfected with an expression vector for the wild-type, the K303R mutant, or co-expressed MTA-L1. The *in vivo* metastatic potential and behavior of the transfected lines will then be examined using both ectopic growth and tail vein injection into athymic nude mice. Metastatic lesions, if present, will be examined for WT ER, K303R ER, and/or MTA-L1 expression using microdissection, genomic sequence, and SNP analyses. The sensitivity of the metastatic lesions to estrogens antiestrogens will be examined to determine their hormonal response to treatment with these agents.

## BODY

### Specific Aim 1

#### **MTA1-L1 inhibits ER $\alpha$ transcriptional activity in human breast cancer cells**

– ER $\alpha$  is a ligand – activated transcriptional factor, to test the effect of MTA1-L1 on ER $\alpha$  activity, we performed the transient transactivation assays. MTA1-L1 was expressed in two commonly used ER $\alpha$  - positive human breast cancer cell lines, namely T47D and MCF-7 cells. Transactivation activity of the endogenous ER $\alpha$  was measured with the vitellogen ERE with TK promoter linked to a luciferase reporter gene (Fig 1, Panel A and B). Upon exposure to the 1 nM E2 for 18-24 hours, the endogenous ER $\alpha$  was activated. In each cell lines, cotransfected MTA1-L1 inhibits the ligand activated endogenous ER $\alpha$  activities to about 50% compared to the vector control group. This data clearly show that MTA1-L1 could inhibit ER $\alpha$  transactivation activity, thus raises the possibility that MTA1-L1 could be an ER $\alpha$  repressor.

**MTA1-L1 overexpression inhibits the anchorage - dependent growth of ER $\alpha$ -positive breast cancer cells** – ER $\alpha$  transcriptional activity is essential for the growth of both normal breast epithelium and many breast tumor cells (4). Since MTA1-L1 inhibits the endogenous ER $\alpha$  transcriptional activity in human breast cancer cells, so we next examined the effect of MTA1-L1 on the proliferation of ER $\alpha$  - positive breast cancer cells. Colony – formation assay has been used to test the effect of ER $\alpha$  repressors such as BRCA1 (5) on the anchorage – dependent growth of ER $\alpha$  - positive breast cancer cells. We thus performed this experiment to test if MTA1-L1 could have any effect on the anchorage – dependent growth of ER $\alpha$ -positive breast cancer cells. We transfected either MTA1-L1 expression vector or the control vector into both MCF-7 and T47D cells, after 14 days selection with G418 in regular MEM media containing 5% FBS, we observed that, compared with the vector control group, overexpression of MTA1-L1 dramatically inhibits the colony formation in both MCF-7 cells and T47D cells (Fig 2, Panel A and B). This result provides another evidence that MTA1-L1 could be an ER $\alpha$  repressor.

**MTA1-L1 is an ER $\alpha$  - binding protein** – The majority ER $\alpha$  cofactors identified so far are ER $\alpha$  - binding proteins, they bind to ER $\alpha$  in either ligand – dependent, as the case of SRC family members or ligand – independent, as the case of MTA1. To analyze whether MTA1-L1 could bind to ER $\alpha$ , we performed glutathion-S-transferase (GST) – pulldown experiment to check the potential in vitro interaction. We incubated the in vitro translated (IVT) ER $\alpha$  or MTA1-L1 with GST-MTA1-L1 or GST-ER $\alpha$  (AF2/Hinge) immobilized glutathione – sepharose respectively (Fig 3, Panel A). There were no signal in the GST-alone immobilized beads; however, we can see their interaction in either GST-ER $\alpha$  or GST-MTA1-L1 (1-254) pulldown lane. Next we examined whether this interaction is present within cells. We performed the coimmunoprecipitation experiments to address this question, firstly we cotransfected Flag tagged MTA1-L1 with either HA-tagged wild-

type or the K303R mutant ER $\alpha$  into HeLa cells, next we employed either protein G sepharose (control) or HA-monoclonal antibody conjugated sepharose to precipitate the ER $\alpha$  complexes, then we performed the western blot analysis using anti-M2 antibody to see if MTA1-L1 is contained in the precipitated ER $\alpha$  complexes. As expected, we observed the MTA1-L1 band in HA-Ab sepharose precipitated complexes but not in the protein G sepharose precipitated complexes, also we observed that the K303R mutation could not block ER $\alpha$  binding with MTA1-L1 (Fig 3, Panel B). Thus, MTA1-L1 is an ER $\alpha$  binding protein both in vivo and in vitro.

**Mapping of the interaction sites between ER $\alpha$  and MTA1-L1** – As we mentioned before, ER $\alpha$  contains at least 4 functional domains. So we next examined the ability of MTA1-L1 to interact with different domains of ER $\alpha$  with GST-pulldown assays. We immobilized equal amount of different GST-ER $\alpha$  fusion proteins on glutathion beads and incubated these beads with equal amount of in vitro translated MTA1-L1 in the absence of hormone. As shown in Fig.4A and its summarization in Figure 4B, MTA1-L1 independently interacts with DBD, Hinge as well as AF2 domain but not AF1 domain. Thus as described for the interaction between ER $\alpha$  and other cofactors such as HET (6), MTA1-L1 potentially interacts with multiple regions of ER $\alpha$ .

Similar to the structure of ER $\alpha$ , MTA1-L1 also contains several function domains (7) as we illustrated in Fig. 4D. The results shown in Fig 2A suggested that either the leucine zipper motif (ELM 2 domain, 234-254 Aas) or the bromo-adjacent domain (BAH domain, 4-166 Aas) or these two domains together can interact with ER $\alpha$ . We used GST-ER $\alpha$  (AF2/Hinge) immobilized glutathion beads to incubate with different fragments of MTA1-L1 synthesized by in vitro translation. The result was shown as Fig. 4C and summarized as Fig. 4D. What we learnt from this result are that, 1) The intact BAH domain of MTA1-L1 can interact with ER $\alpha$ ; 2), ELM 2 motif is not essential for the interaction of MTA1-L1 with ER $\alpha$ . But we still cannot eliminate the possibility whether other MTA1-L1 motifs could interact with ER $\alpha$ .

**Ligand binding does not block the interaction between ER $\alpha$  and MTA1-L1** – The AF2 domain contains a well conserved amphipathic  $\alpha$ -helix (helix 12) structure, which has been shown to be essential for the ligand-inducible AF2 function. Upon ligand binding, this helix will fold back and generate a transcriptionally active conformation (8). Since the results shown in Fig. 4A demonstrated that MTA1-L1 can bind to the ER $\alpha$  AF2 domain, so we next investigated whether ligand-occupying status of ER $\alpha$  would affect this interaction. We performed GST-pulldown as well as coimmunoprecipitation experiments to address this question, the results as shown in Fig. 5 (Appendice, Panel A and B) demonstrated that E2 treatment does not interfere with this interaction either in vitro or in vivo. Figure 5A also demonstrated that the K303R mutation does not alter this interaction regardless ligand-occupying status.

**MTA1-L1 is an insufficient repressor of K303R ER $\alpha$  mutant** - MTA1-L1 is a component of the core complexes of NuRD (9) and inhibits the transcriptional activity of wild type but not a p53 mutant harboring mutation in potential acetylation sites (10). This raises the possibility that MTA1-L1 only acts on the acetylated or acetylatable p53. As we demonstrated before (11), K303R mutation disrupts the major acetylation site of ER $\alpha$ , so that this ER mutant mimics the de- or hypoacetylated ER $\alpha$ . Thus we hypothesized that

MTA1-L1 is an insufficient repressor of K303R ER $\alpha$  mutant as compared with wild-type ER $\alpha$ . We then performed the transient transactivation assays to address this hypothesis. We cotransfected MTA1-L1 expression vectors with either wild type or the K303R mutant ER $\alpha$  expression vectors in both MCF-7 and T47D cells. As we predicted that, overexpression of MTA1-L1 represses wild-type ER $\alpha$  activity by about 50%, but it cannot inhibit the coexpressed K303R ER $\alpha$  mutant transcriptional activity ( Fig. 6, Panel A and B).

## **KEY RESEARCH ACCOMPLISHMENTS**

1. Identification of MTA1-L1 as an ER $\alpha$  co-repressor which inhibits ER transcriptional activity in various cell lines and inhibits ER-positive cells proliferation
2. Identification of MTA1-L1 as an insufficient co-repressor for K303R ER mutation
3. Mapping the interaction domains between ER and MTA1-L1

## **REPORTABLE OUTCOMES**

1. (2) Meeting abstracts included in the Appendix
2. Figures and relevant figure legends as well as development of plasmid constructs were included in Appendix

## CONCLUSIONS:

We have successfully completed the major part the Aim 1. Results on this project have been resulted in two meeting abstracts. Exciting results ~~describing~~ this ER mutation were generated. The work and findings from this fellowship award will continue to be studied in the laboratory of the mentor, Suzanne A. W. Fuqua. Undoubtedly, the funds for this fellowship have been well spent with much success. It is also conceivable that this line of research will lead to direct translational benefit.

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## APPENDICES

Reprint 1: Cui, Y, Hopp T, Zhang M. Fuqua SAW : A naturally occurring point mutation alters ER $\alpha$  crosstalk with regulatory signaling pathways. Breast Cancer Research and Treatment. 76, S54, 2002

Reprint 2: Yukun Cui, Mao Zhang, Torsten Hopp, Wei Gu, and Suzanne Fuqua. Metastasis-associated Protein 1-like 1 (MTA1-L1) is an estrogen receptor alpha binding protein and modulates ER alpha activity. Proceedings of the American Association for Cancer Research 43, 117(#586), 2002

**Construction of Plasmids** - GST-fused AF2 /Hinge domain harboring K303R mutant were generated with the same primers as we generated GST-fused AF2 plus Hinge domain with HA-K303R ER $\alpha$  as template, the GST-fused Hinge domain (253-310) with or without K303R mutation were generated through PCR and inserted into pGEX 4T-1 vector (Pharmacia, Piscataway, NJ) through Bam HI and EcoRI sites. For the GST fusion vectors of MTA1-L1, Full length MTA1-L1 cDNA were generated by performing RT-PCR with the RNA from MB-MDA-231 cells as template, the cDNA was then cut by XhoI (5') combined with Asp718 or EcoR V and inserted into pGEX 4T-3 vector through XhoI and Not I (blunted with DNA polymerase I large fragment). The MTA1-L1 vectors used for in vitro translation were cut from Flag-MTA1-L1 by Nde I (5') combined with BamHI (full length), Asp718 (1-253), Hind III (1-237), or EcoR V (1-115) and inserted into pET-15b (Novagen, Madison, WI) through Nde I and Bam HI sites.

### Figure Legends

**Fig.1. MTA1-L1 inhibits ER $\alpha$  transcriptional activity in human breast cancer cells.** Cotransfection of 1  $\mu$ g ERE-tk-Luc with 0.8  $\mu$ g expression vector of either MTA1-L1 or empty vector into MCF-7 (Panel A) or T47D (Panel B) and treated with  $10^{-9}$  M E2 for 18-24 hours. Luciferases were normalized to the cotransfected internal control of pCMV- $\beta$ -gal. The data shown as the fold induction to E2 treatment and represents the mean  $\pm$  SEM for 9 separate transfections.

**Fig.2. MTA1-L1 overexpression inhibits the anchorage – dependent growth of ER $\alpha$  - positive breast cancer cells.** 5  $\mu$ g either MTA1-L1 expression plasmid or control vectors were transfected into each 100 mm tissue culture plates of MCF-7 (Panel A) or T47D (Panel B) cells. 24 hours later, cells were selected by MEM containing 5% FBS supplemented with 800  $\mu$ g/ml G418 for 14 days. The colony numbers were then counted. The data shown is the relative colony numbers normalized to the vector control group and represents the mean  $\pm$  SEM for 2 separate experiments

**Fig. 3. MTA1-L1 is an ER $\alpha$  binding protein.** (A). In vitro interaction. ER $\alpha$  (IVT) and MTA1-L1 (IVT) were labeled with  $^{35}$ S-methionine by in vitro translation and tested for interaction with GST-MTA1-L1 and GST-ER $\alpha$  respectively in the absence of ligand. (B). *In vivo* interaction. Hela cells were transiently transfected with expression

vectors for either HA-tagged wild type (Wt) or K303R mutant ER $\alpha$  (K303R) with Flag-tagged MTA1-L1. The cell lysates were immunoprecipitated with either anti-HA monoclonal antibody conjugated sepharose or protein G sepharose (negative control). The immunoprecipitates were then subjected to electrophoresis. Anti-M2 antibody was used to detect the immunoprecipitated MTA1-L1 by immunoblot.

**Fig. 4. Mapping of the interaction sites between ER $\alpha$  and MTA1-L1.** (A). Mapping of ER binding sites in MTA1-L1. Different fragments of MTA1-L1 as indicated in the figure were labeled with  $^{35}\text{S}$  – methionine by in vitro translation and test for interaction with GST-ER $\alpha$  in the absence of ligand. (B). Illustration of MTA1-L1 structures and summarization of the result shown in Fig. 4A. (C). Mapping of MTA1-L1 binding sites in ER $\alpha$ . Full length MTA1-L1 was labeled with  $^{35}\text{S}$  – methionine by in vitro translation and test for interaction with equal amounts of different GST-ER $\alpha$  in the absence of ligand. (D). Illustration of ER $\alpha$  structure and summarization of the result shown in Fig. 4C.

**Fig. 5. Ligand binding does not block the interaction between ER $\alpha$  and MTA1-L1.** (A). Full length MTA1-L1 was labeled with  $^{35}\text{S}$  – methionine by in vitro translation and test for interaction with equal amounts of either GST-wtER $\alpha$  or GST-K303R ER $\alpha$  in the absence or presence of  $10^{-6}$  M E2. (B). Hela cells were transiently transfected with expression vectors for HA-tagged wild type (Wt) with Flag-tagged MTA1-L1. 24 hours later the cells were treated by  $10^{-9}$  M E2 for another 24 hours. The cell lysates were immunoprecipitated with either anti-HA monoclonal antibody conjugated sepharose or protein G sepharose (negative control) in the absence or presence of  $10^{-9}$  M E2. The immunoprecipitates were then subjected to electrophoresis. Anti-M2 antibody were used to detect the immunoprecipitated MTA1-L1 by immunoblot.

**Fig. 6. MTA1-L1 is an insufficient repressor of K303R ER $\alpha$  mutant.** Cotransfection of 1  $\mu\text{g}$  ERE-tk-Luc, 25 ng expression vector of either wtER $\alpha$  or K303R ER $\alpha$  with 0.8  $\mu\text{g}$  expression vector of either MTA1-L1 or empty vector into MCF-7 (Panel A) or T47D (Panel B) and treated with  $10^{-9}$  M E2 for 18-24 hours. Luciferases were normalized to the cotransfected internal control of pCMV- $\beta$ -gal. The data shown as the fold induction to E2 treatment and represents the mean  $\pm$  SEM for 9 separate transfections.

168 A naturally occurring point mutation alters ER $\alpha$  crosstalk with regulatory signaling pathways.

Cui Y, Hopp T, Zhang M, Fuqua SAW. Baylor College of Medicine, Houston, TX

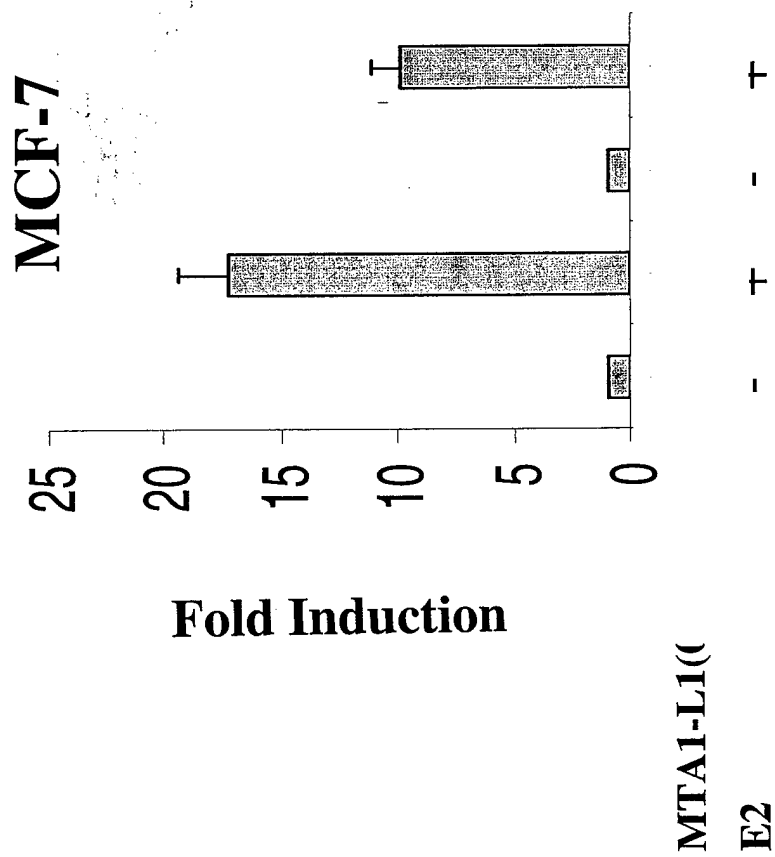
Estrogen receptor  $\alpha$  (ER $\alpha$ ) belongs to the superfamily of ligand-dependent transcription factors. It is well accepted that in addition to being activated by ligand binding, ER $\alpha$  activity is also regulated by diversified signaling pathways, such as that of Histone Acetyl Transferase and G-proteins. Recently we have isolated a somatic ER $\alpha$  mutation in human breast hyperplasias and invasive breast cancers. This mutation (Lys 303 to Arg, K303R) disrupts the major acetylation site of ER $\alpha$  at K303. Since we have shown that this mutation alters ER $\alpha$  interactions with the p160 family of ER coactivators, we hypothesized that it might also alter ER $\alpha$  crosstalk with other signaling effectors. We found that wildtype ER $\alpha$  activity could be inhibited by a component of the nucleosome remodeling and histone deacetylase complexes, called metastasis-associated protein 1-like 1 (MTA1-L1) using transient transactivation assays. Furthermore, we found that the activity of the K303R ER $\alpha$  mutant is refractory to MTA1-L1 inhibition. We also determined that MTA1-L1 could enhance deacetylation of wildtype ER $\alpha$  using in vitro deacetylation assays. Finally we demonstrated that a selective HDAC inhibitor, sodium butyrate could restore MTA1-L1 repressed wildtype ER activity. Taken together, our results suggest that the K303R mutation alters the crosstalk between ER $\alpha$  and diversified ER $\alpha$  pathways that normally downregulate ER signaling. We hypothesize that this loss of regulation may provide additional selective forces for ER-mediated tumor progression.

**#586 Metastasis-associated protein 1-like 1 (MTA1-L1) is an estrogen receptor alpha binding protein and modulates ER alpha activity.** Yukun Cui, Mao Zhang, Torsten Hopp, Wei Gu, and Suzanne A. W. Fuqua. *Baylor college of medicine, Houston, TX, and Columbia University, New York, NY.*

The estrogen receptor alpha (ER alpha) belongs to the superfamily of ligand-dependent transcription factors and in addition to being activated by ligand binding, its activity is also influenced by various modifications including phosphorylation and mutation. Recently we have isolated a somatic ER alpha mutation in human breast hyperplasias. This mutation occurs in the major p300 induced acetylation site of ER alpha (Lys 303 to Arg, K303R), and is significantly correlated with both breast cancer genesis and development. Previously, studies show that the ER alpha mutation is hypersensitive to very low levels of estradiol (1 pM). We report here that a component of the nucleosome remodeling and histone deacetylase complexes (NuRD), called metastasis-associated protein 1-like 1 (MTA1-L1), is an ER alpha binding protein demonstrated by both GST-pull down and coimmunoprecipitation. Also, we found that this interaction is ligand independent and requires the N-terminal region of MTA1-L1. Furthermore, we show that MTA1-L1 downregulates wild-type ER alpha, but not the K303R ER alpha mutation activities in transient transactivation assays. Taken together, our results suggest that the altered ER  $\alpha$ -containing NuRD function due to the K303R mutation could be a possible mechanism for this hypersensitive phenomenon.

Fig 1

A



B

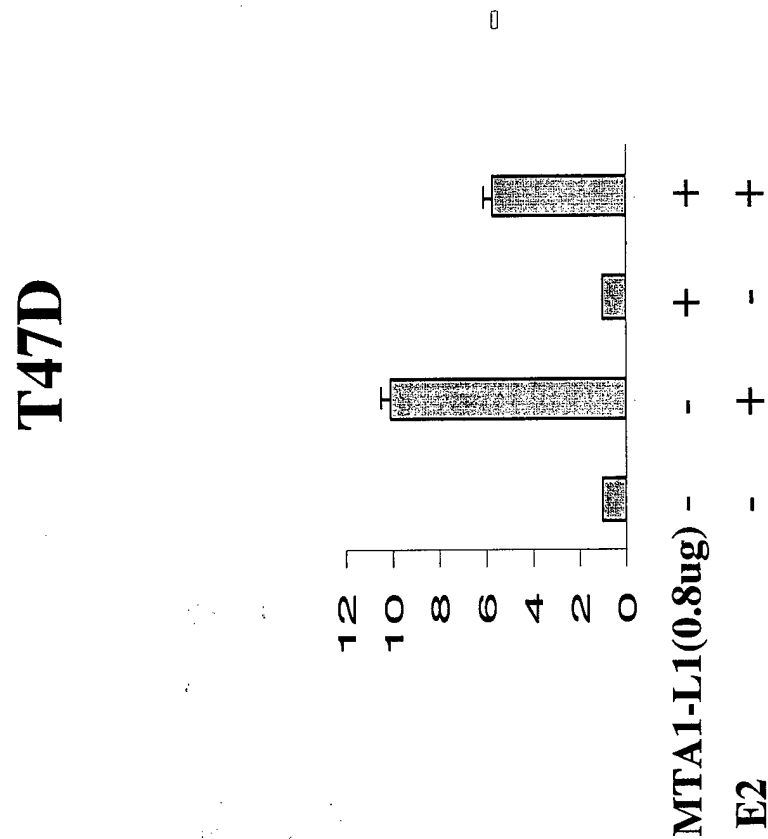


Fig. 2

A

Relative Colony  
Formation

MCF-7

120  
100  
80  
60  
40  
20  
0



Vector MTA1-L1

B

T47D

120  
100  
80  
60  
40  
20  
0



Vector MTA1-L1

Fig. 3

A

20% input  
GST  
MTA1-L1(1-234)  
MTA1-L1(1-416)



ERα IVT

20% input  
GST  
ERα



MTA1-L1 IVT

B

IP

4% input  
Con ER

Wt-ER K303R ER  
Wt-ER K303R ER  
Wt-ER K303R ER  
Wt-ER K303R ER

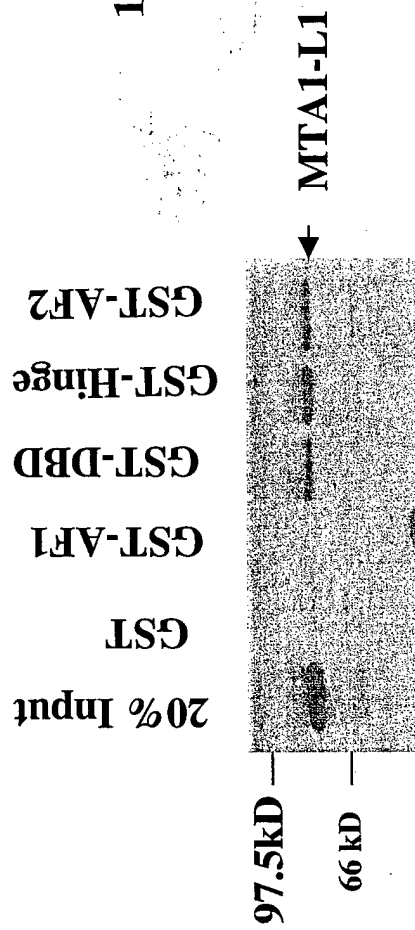
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MTA1-L1

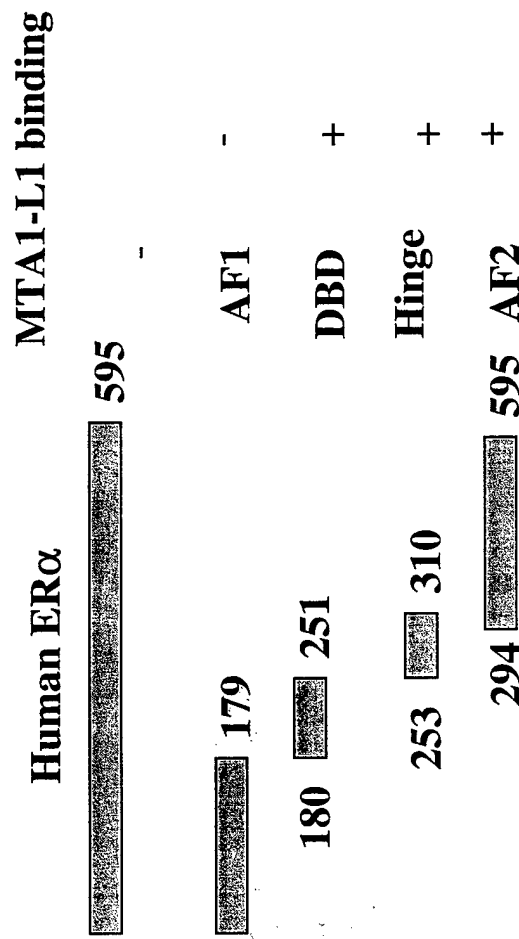


Fig. 4

A



B





C

Input		GST-pull down	
		GST	
		GST-ER(251-595)	
		MTA1-L1	
220	—	Full Length	Full Length
97.5	—	1-254	1-254
66	—	1-237	1-237
46	—	1-116	1-116
30	—	Full Length	Full Length
21	—	1-254	1-254
14	—	1-237	1-237
	—	1-116	1-116

D

668

4 166 231 253 265 323



BAH ELM2 SANT

ER Binding

668

4



+

254



+

237



+

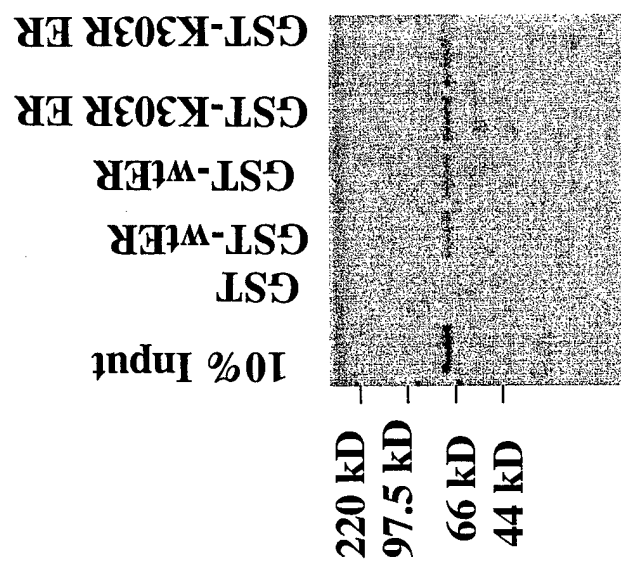
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Fig. 5

A



E2

- + - +

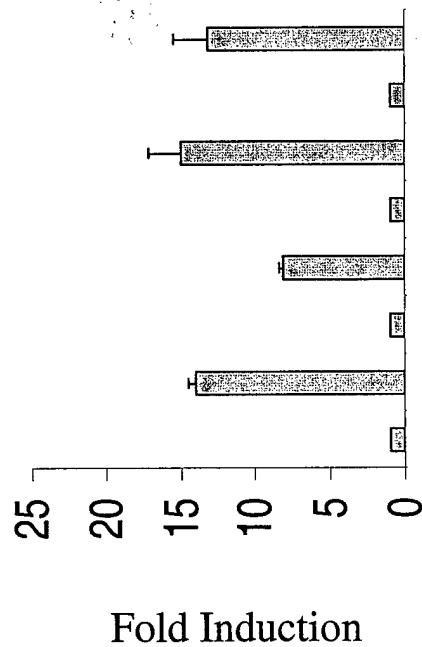
B



Fig. 6

A

MCF-7

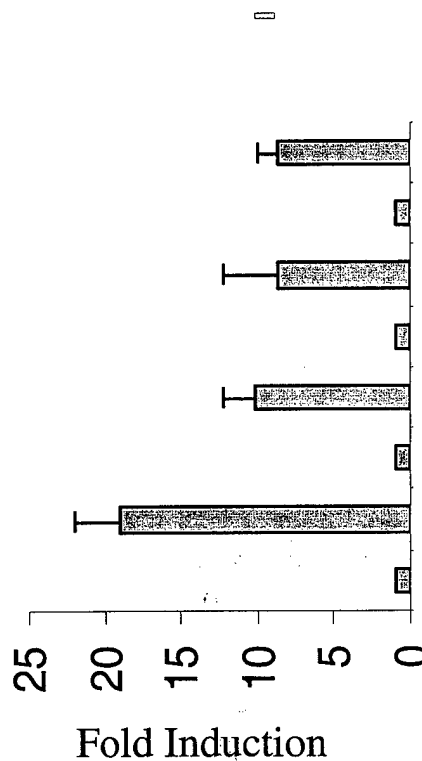


Fold Induction

WT-ER  
K303R  
E2  
MTA2

B

T47D



Fold Induction

WT-ER  
K303R  
E2  
MTA2